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PAK'n It In: Identification of a Selective PAK Inhibitor

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p21-activated kinases are important signaling kinases for which no specific chemical inhibitors are known. In this issue of *Chemistry & Biology*, Deacon et al. target allosteric transitions undergone during PAK activation to identify a selective inhibitor (Deacon et al., 2008).

The p21-activated kinases (PAKs) are important mediators of Rho GTPase signaling, and are implicated in biological processes ranging from cytoskeletal dynamics and motility to tumorigenesis and apoptosis (Bokoch, 2003; Arias-Romero and Chernoff, 2008). The Group I PAKs (PAKs1–3) are regulated by an autoinhibitory mechanism that is relieved by the binding of active Rac and Cdc42 GTPases to an N-terminal recognition domain. The binding of other regulatory proteins, sphingolipids, and the kinase-mediated phosphorylation of specific sites within the PAK N terminus can release PAK autoinhibition as well.

Most of the biological activities of Group I PAKs have been determined using the autoinhibitory domain contained within aa 83–149 of PAK1 expressed *in trans* as a means to inhibit the Group I PAKs. While extremely useful, there are potential problems inherent in this approach. These include the need to introduce the autoinhibitory peptide fragment into cells, and the potential for this region to bind unintended targets, including Rac or Cdc42, and possibly other proteins (Thullberg et al., 2007). Likewise, PAKs have scaffolding functions that affect cell signaling, apart from their action as kinases. Thus, depletion of PAK proteins using siRNA not only affects substrate phosphorylation, but also localization of proteins to the PAK scaffold.

In general, the highly conserved ATP binding pockets of kinases have been amenable to the identification of small molecular weight inhibitors that compete for ATP binding. The PAKs have been a major exception because screening efforts for such competitive inhibitors have, to this author's knowledge, not been successful. In this issue of *Chemistry & Biology,* Peterson and colleagues use a screening approach based on the identification of allosteric inhibitors of the conformational changes that PAK undergoes during activation to identify PAK inhibitors (Deacon et al., 2008).

Many current inhibitor screens make use of constitutively activated forms of the kinase and target enzyme active sites. In nature, however, protein function is often regulated through allostery, a phenomenon whereby the binding of a small molecule to one site regulates the activity of another, remote, site on the protein. Allosteric regulators act by altering a conformational equilibrium of the protein between active and inactive states. Such equilibria can also be targeted by nonnatural small molecules that perturb these conformational transitions. The approach of targeting allosteric transition states during protein activation has been successfully used to identify inhibitors of other enzymes, e.g., Wiskostatin, an N-WASP inhibitor that stabilizes the autoinhibited conformation of this cytoskeletal-regulatory enzyme (Peterson et al., 2004). In addition to providing alternative avenues for inhibiting enzymes, this approach, in principle, could allow targeting of allosterically regulated proteins that lack catalytic sites. Indeed, many clinically important signaling pathways are mediated by proteins lacking catalytic activity that serve instead as scaffolding, structural, or regulatory components. Endogenous protein inhibitors (e.g., the tumor suppressor Merlin) or activators (e.g., CIB1) of PAK1 kinase activity that target the PAK1 autoregulatory strategy have been described, suggesting that modulation of the autoregulatory domain may be a normal physiological strategy for modulating PAK catalytic activity.

The primary screen of a 33,000 small molecular weight compound library performed by Deacon et al. was based on the catalytic activity of full-length PAK1 activated by the allosteric modulator Cdc42-GTPγS. This screen identified 342 compounds able to inhibit PAK activity by greater than 3 standard deviations below the control mean. A secondary screen was conducted in the presence of 1 mM ATP to minimize detection of ATP-competitive inhibitors, reducing the final number of active inhibitors to 32. 2,2′–dihydroxy-1,1′–dinaphthyldisulfide (termed IPA-3 for allosteric *i*nhbitor targeting *PA*K1 activation-*3*), a highly active compound with an IC_{50} of 2.5 μM, was chosen for further characterization.

IPA-3 was shown not to act via competition with PAK1 ATP binding, nor did it (as a disulfide) target exposed cysteine residues on PAK1. Inhibition was observed when either Cdc42-GTPγS or sphingosine was used as an enzyme activator. Interestingly, order-of-addition experiments demonstrated a dramatic reduction in the efficacy of IPA-3 when added after PAK1 preactivation and autophosphorylation had occurred.

In the autoinhibited conformation, the activation loop (aa 408–428) of PAK1 is folded into the catalytic site, thereby masking the activation loop and preventing access to the critical Thr423 residue (Figure 1). Upon relief of autoinhibition, a conformational change in the activation loop exposes Thr423 to phosphorylation by other PAK1 monomers, as well as other kinases (Chong et al., 2001; Zenke et al., 1999). Deacon et al. demonstrate that IPA-3 does not stabilize the native autoinhibited conformation of PAK1, as originally suspected, but actually promotes an inactive conformation of PAK1 in which Thr423 is exposed (see Figure 1). Indeed, PAK1 could be phosphorylated at Thr423 by added exogenous kinases, but was not phosphorylated at that site by PAK1 itself. Phosphorylation of Thr423 by exogenous kinase did not overcome

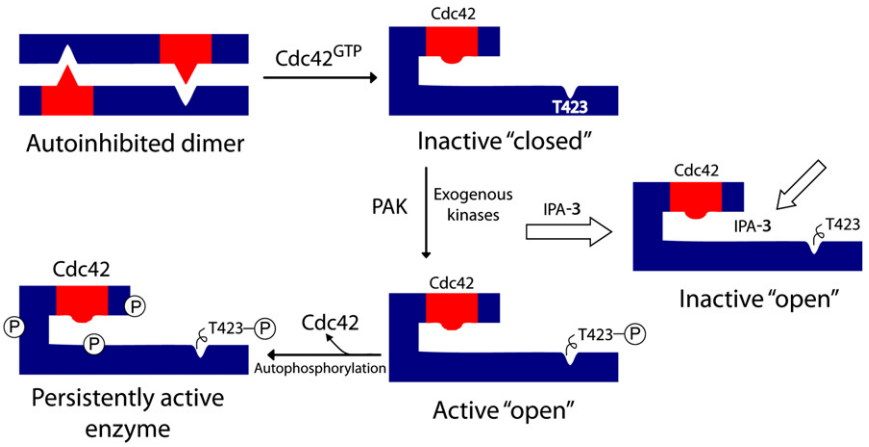

Figure 1. A Schematic Representation of Steps in the Pak1 Autoinhibition Cycle

The autoinhibition of Pak1 is mediated by formation of an inactive homodimer in which the autoregulatory region (red) of one monomer binds and inhibits *in trans* the catalytic domain of its partner, and vice versa (Lei et al., 2000). The kinase inhibitory segment of the autoregulatory region (indicated by triangular shape) binds in the activation site cleft and sequesters the kinase activation loop in an inactive conformation. The binding of active GTPase to a region partially overlapping the autoinhibitory region causes dissociation of the PAK dimer and displacement of the inhibitory interaction through destabilization of the inhibitory domain (indicated by change in shape). This results in a transient inactive "closed" enzyme conformation. This conformation quickly shifts to an "open" conformation in which the T423 site in the activation loop is exposed, resulting in its rapid phosphorylation, either by PAK monomers acting "*in trans*" or by exogenous kinases such as PDK1, and enzyme activation. IPA-3 induces (or stabilizes) a unique state characterized by an open conformation in which T423 is exposed, yet PAK1 remains inactive. Autophosphorylation events at multiple sites within PAK1 stabilize the catalytically competent, monomeric conformation (Chong et al., 2001; Zenke et al., 1999).

the inhibitory effect of IPA-3, however. Whether IPA-3 induces this catalytically inactive conformation of PAK1, or stabilizes a previously unsuspected transition intermediate distinct from the autoinhibited conformation, is unknown. A precise understanding of the inhibitory mechanism will necessitate detailed structural analyses.

While all three Group I PAKs were inhibited by IPA-3, the Group II PAKs, with catalytic domains that are highly conserved with those of the Group I PAKs but which are not regulated by an autoregulatory strategy (Arias-Romero and Chernoff, 2008), were insensitive to IPA-3. Indeed, the high degree of specificity of the IPA-3 inhibitor was particularly impressive—only 9 of 214 kinases tested (4% of total) were inhibited by more than 50% at 10 μM IPA-3. In comparison, the highly selective Bcr-Abl inhibitor imatinib inhibited 12% of kinases tested in the same assay. Efficacy and selectivity of IPA-3 toward PAK1 was also observed in intact cells. Specificity profiling of clinical kinase inhibitors has revealed that many "selective" compounds also target seemingly unrelated kinases (Karaman et al., 2008). IPA-2 achieves a degree of selectivity comparable to that of the highly specific clinical inhibitors imatinib and gelfitinib. Thus, IPA-3 is a unique Group I PAK inhibitor that achieves a high degree of specificity by targeting the distinct autoregulatory mechanism used by the Group I PAKs.

The present study provides an excellent demonstration of the potential utility of screening for allosteric agents for the identification of novel inhibitors of enzymes that have proven recalcitrant to conventional screens based upon targeting of the catalytic site. The availability of selective chemical inhibitors of PAKs will hopefully help to resolve confounding issues raised by the absence of cytoskeletal and other defects in PAK1 knockout mice. It has been hypothesized that the activity of other Group I PAKs may compensate for the loss of PAK1. Likewise, substantial data have implicated PAK1 in processes such as cancer and metastasis (Kumar et al., 2006), and in neuronal mechanisms contributing to mental retardation and neurodegenerative diseases (Boda et al., 2006). Inhibitors of Group I PAKs have been suggested as a potential basis for clinical therapy in such conditions. The identification of IPA-3 and related compounds provides a long-awaited opportunity to test these hypotheses.

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